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Neural Crest Formation in *Xenopus laevis:* Mechanisms of *Xslug* Induction

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A study of the induction of the prospective neural crest in *Xenopus laevis* embryos has been carried out, using the expression of *Xslug* as a specific marker for the neural crest. We have analyzed the competence and the specification of the neural crest. The competence to express *Xslug* was analyzed using two different approaches: (1) *in vitro* culture of conjugates of dorsal mesoderm and ectoderm taken from embryos at different ages and (2) grafts of equivalent pieces of ectoderm in the neural fold region of a gastrula or a neurula. Similar results were obtained with both methods: the ectoderm loses the competence to respond to neural fold induction at the end of gastrulation. Neural crest specification was analyzed by culturing a region of the ectoderm that contained the prospective neural crest and analyzing *Xslug* expression. Our results show that neural folds are specified autonomously to express *Xslug* by the end of gastrulation. By grafting labeled neural plate into lateral epidermis we have shown that neural crest can be induced by an interaction between neural plate and epidermis. Furthermore, neural crest cells come from both tissues. We have discarded the possibility that these neural crest cells are induced by a signal coming from the underlying lateral plate, by a homeogenetic signal coming from the host neural plate, or by regeneration of crest cells from the dissected neural plate. We propose a model to explain how the neural crest cells are induced at the border of the neural plate in *X. laevis.*

INTRODUCTION

During the process of neurulation in amphibia, dorsal ectodermal cells thicken to form a keyhole-shaped neural plate. At the lateral margins of the neural plate, changes in cell shape and interactions with surrounding tissue combine to generate the neural folds. These subsequently rise and fuse at the dorsal midline, converting the neural plate into a cylindrical neural tube that gives rise to the central nervous system (CNS). A population of cells called the neural crest migrate from the neural folds and form a wedge-shaped cell mass lying above the closed neural tube (Schroeder, 1970). The crest cells remain at this position for a short time before migrating through the embryo along defined pathways and differentiating into a wide range of neuronal and nonneuronal cell types.

Several hypotheses have been proposed to explain how neural crest cells are generated from the ectoderm. Raven and Kloos (1945) have postulated the existence of a combined CNS/neural crest ''evocator'' molecule that is produced at different concentrations across the archenteron roof. Medially, a high concentration of evocator induces CNS, while lower concentrations laterally induce the formation of neural crest cells. A second model, proposed by Albers (1987), postulates the existence of a single neural-/neural crest-inducing molecule that spreads through the ectoderm from the dorsal midline to more ventral regions of the embryo. According to this model, the position of the neural folds is determined by temporal changes in the competence of the ectoderm. At early stages, (dorsal) ectoderm responds to the emanating signal by forming neural tissue. As development proceeds, the ectoderm loses competence to form neural plate, and during a short phase of ''weak'' competence the ectoderm responds to the signal by forming neural fold material, including prospective neural crest cells. A short time later, the (lateral) ectoderm has lost all competence to respond to the signal and the ectoderm subsequently differentiates as epidermis. Although there is evidence that supports this model in accounting for the size of the neural plate (Albers, 1987; Servetnick and Grainger, 1991), no one has tested it for the induction of the neural folds or neural crest cells. Many recent experiments favor a third hypothesis which proposes that neural crest cell formation requires interactions between neural plate and

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nonneural ectoderm (Moury and Jacobson, 1989, 1990; Zhan and Jacobson, 1993; Dickinson *et al.*, 1995; Liem *et al.*, 1995; Mayor *et al.*, 1995; Selleck and Bronner-Fraser, 1995).

To ascertain which of these models best describes the formation of the neural crest *in vivo*, it is necessary to investigate temporal changes in the competence of ectoderm to form neural crest cells and the time during development when ectodermal cells become committed to a neural crest fate. Unfortunately, such experiments have been hindered by the lack of reliable early markers for neural crest cells. Recently, a number of genes have been cloned in *Xenopus* that are expressed very early in the prospective neural folds and might therefore serve as markers for neural crest cells. These include the zinc-finger gene *Xsna* (the *Xenopus* homologue of the *Drosophila snail;* Mayor *et al.*, 1993; Essex *et al.*, 1993) and the helix-loop-helix gene *Xtwi* (the *Xenopus* homologue of the *Drosophila twist;* Hopwood *et al.*, 1989; Essex *et al.*, 1993). Unfortunately, both these genes are also expressed in mesodermal cells around the same time of development. In contrast, *Xslug* (the *Xenopus* homologue of the chicken zinc-finger gene *Slug;* Nieto *et al.*, 1994) does appear to be a useful marker because at early (neurula) stages (stage 12 until stage 17), expression is restricted to prospective neural crest (Mayor *et al.*, 1995).

We have used expression of the gene *Xslu* to investigate early events in the ontogeny of neural crest cells in *Xenopus*. Specifically, we have attempted to ascertain (i) the stages at which neural plate is competent to form neural crest cells, (ii) the time at which ectodermal cells become committed to a neural crest fate, and (iii) whether neural plate–epidermis interactions can induce the formation of neural crest cells. Our results suggest that (i) ectoderm can express *Xslug* in response to signals from dorsal mesoderm until stage 12, but remains competent to express a neural marker (*Xsox-2*) after this time, (ii) ectoderm cells become committed to a neural crest fate between stages 11 and 13, (iii) the neural crest marker *Xslug* can be induced by an interaction between neural plate and epidermis, and (iv) the crest cells induced at the border of the neural plate and epidermis originate from both the neural plate and the epidermis. Finally, we propose a model to explain the induction of the neural crest at the border of the neural plate in *Xenopus* embryos.

MATERIALS AND METHODS

Embryos and Explants

Xenopus embryos were obtained by artificial fertilization, dejellied in 2% cysteine (Smith and Slack, 1983), reared in 10% normal amphibian medium (NAM; Slack, 1984), and staged according to Nieuwkoop and Faber (1967). Explants of different tissues were isolated using glass microneedles or eyebrow knives. All the dissections and transplants were performed in 75% NAM and after healing, embryos or tissues were cultured in 38% NAM. To mark the prospective neural plate border for specification experiments, the animal caps were stained to include only the prospective folds, as described in Mayor *et al.* (1995). Briefly, to mark the prospective neural plate border, animal caps were stained with Nile blue so that exactly the same region was used at each point. At each time point, the labeled cap was dissected from the embryo at stage 10. At each time point, the labeled cap was dissected from the embryo and freed of mesoderm and the dorsal half was used for the specification experiments.

LFDx or LRDx Injection

Dejellied 1-cell embryos were placed in 75% NAM with 5% Ficoll, injected with 10 nl of 25 mg/ml solution of lysinated-fluorescein dextran or lysinated-rhodamine dextran (LFDx and LRDx, respectively; Molecular Probes) and subsequently reared at 14°C as described above.

Transplantation of Tissues and Conjugates

Tissues were transplanted from LFDx- or LRDx-labeled donors to the prospective neural fold region or lateral epidermis of unlabeled hosts, as described under Results. Transplants were held in place during healing with small curved glass bridges (Henry and Grainger, 1987). Conjugates of ectoderm with mesoderm or epidermis with neural plate were prepared as described by Nieuwkoop (1969).

Whole-Mount in Situ Hybridization

Sense- and antisense-containing RNA probes were prepared for the *Xslug* (Mayor *et al.*, 1995) and *Xsox-2* (kindly provided by Dr. R. M. Grainger) genes. Specimens were prepared, hybridized, and stained by the method of Harland (1991), with the modifications described in Mayor *et al.* (1995) and the changes introduced by T. Doniach (S. Gould and R. Grainger, personal communication). Basically, the RNase treatment was omitted and the antibody was incubated and washed in maleic acid buffer (100 m*M* maleic acid, Sigma; 150 m*M* NaCl, pH 7.5), containing 2% of Boehringer Mannheim blocking reagent.

Preparation of Sections

After *in situ* hybridization, LFDx-labeled embryos were postfixed in 4% formaldehyde, dehydrated through an ethanol-xylene series, embedded in wax, and sectioned at 12 μm. Sections were collected on slides and viewed and photographed using epifluorescence optics (Zeiss). The sections were photographed under white light for comparison with fluorescent images.

RESULTS

The Competence of the Ectoderm to Respond to Neural Crest-Inducing Signals from the Mesoderm Is Lost at the End of Gastrulation

We have started our analysis of neural crest induction by trying to identify the time during development when ectoderm is competent to respond to neural crest-inducing signals from the mesoderm. We have studied the competence of the ectoderm using two different approaches.

In vitro analysis of ectodermal competence. We have shown previously that dorsal mesoderm and lateral mesoderm from stage 10.5 embryos have the ability to induce the neural crest marker *Xslug* in animal caps (Mayor *et al.*, 1995). However, this study did not determine the stages at which ectoderm is competent to respond to the mesodermal signal. To this end, we have combined pieces of dorsal mesoderm from stage 10.5 embryos with ventral animal caps isolated from embryos at different stages of development (stage 10 to 13; Fig. 1a). The conjugates were subsequently cultured to an equivalent of stage 17 in unoperated embryos, a stage at which *Xslug* is highly expressed. The conjugates were fixed and examined by whole-mount *in situ* hybridization using an *Xslug* antisense probe.

Animal caps or dorsal mesoderm cultured alone did not express *Xslug*, regardless of the time of isolation (Fig. 1t). In contrast, expression of *Xslug* was observed after combining these tissues. Expression was very strong when the ectoderm was isolated from early gastrula-stage embryos (stage 10, Fig. 1d and stage 11, Fig. 1e) and all the examined explants showed *Xslug* induction (Table 1, row Conjugate *Xslug*). However, *Xslug* expression decreased significantly when the ectoderm was taken from embryos at more advanced stages (stage 12, Fig. 1f, Table 1), and could not be detected when ectoderm was isolated from embryos at the end of gastrulation (stage 13, Fig. 1g, Table 1). These results suggest that the competence to respond to neural crest-inducing signals from the mesoderm is lost at the end of gastrulation, around stage 12.

In our assay, the formation of neural crest cells may be a consequence of the dorsal mesoderm first inducing neural tissue in the animal caps. If this is so, the absence of *Xslug* expression in older animal caps may reflect a decline in their competence to respond to neural-inducing signals rather than neural crest-inducing signals. In order to investigate this possibility, we have performed animal cap/dorsal mesoderm co-cultures to determine the changes in competence of the animal caps to express the neural plate marker *Xsox-2* (R. Grainger, personal communication; Fig. 1h). When a piece of ectoderm was cultured in isolation until stage 17 it never expressed the *Xsox-2* gene (Fig. 1i). However, when the same tissue was cultured with dorsal mesoderm, a clear *Xsox-2* expression was detected (Figs. 1j and 1k; Table 1, row Conjugate *Xsox*), and we find that the competence of animal caps to express *Xsox-2* is maintained until stage 14/15 (not shown). We conclude that the loss of *Xslug* expression at stage 12–13 is not due to a decrease in the competence of animal cap to respond to neural-inducing signals but instead reflects a real change in the ability to form neural crest cells. We have also performed experiments to investigate whether there are differences between dorsal ectoderm and ventral ectoderm in their ability to respond to neural crest-inducing signals from dorsal mesoderm. No difference in the ability of these two tissues to express *Xslug* was detected (Table 1).

In vivo analysis of ectodermal competence. The experiments discussed above were designed to investigate the interactions occurring between ectoderm and mesoderm only. However, we cannot exclude the possibility that additional signals are involved in neural fold induction *in vivo*. In order to preserve all the possible inductive interactions present in a normal embryo, we have investigated the competence of animal cap ectoderm by grafting it into the prospective neural fold regions of host embryos (Fig. 1b).

In a first set of experiments, stage 13 embryos were used as hosts because their neural folds are clearly visible. Ectodermal tissue was taken from the ventral region of an embryo previously injected with LRDx. The grafted embryos were allowed to heal in 75% NAM for a few hours, cultured in 38% NAM until stage 17, fixed, and analyzed for *Xslug* expression by whole-mount *in situ* hybridization. Similar to our *in vitro* experiments, we found that ectoderm isolated

FIG. 1. Analysis of the competence of the ectoderm to respond to neural fold and neural plate induction. (a, d–g) Ventral animal caps were taken at different stages as indicated in the figure and combined with a piece of dorsal mesoderm taken from stage 10.5 (yellow in a). The conjugates were cultured until stage 17, fixed, and examined by whole-mount *in situ* hybridization using an *Xslug* antisense probe. (d) Stage 10 ectoderm; notice the strong *Xslug* induction (arrows). (e) Stage 11 ectoderm; notice the *Xslug* induction (arrows). (f) Stage 12 ectoderm; notice the weak *Xslug* induction (arrows). (g) Stage 13 ectoderm; no induction was detected, although it should be noticed that this photograph was taken under different magnification and light conditions than in (d–f). (h–k) Similar experiment to that described in (a) but *Xsox-2* expression was analyzed. (h) Control embryo showing *Xsox-2* expression in the neural plate. (i) Control of ectoderm taken from a stage 10 embryo, cultured *in vitro* until stage 17, fixed, and analyzed for *Xsox-2* expression. No expression was detected. (j) Stage 12 and (k) stage 13 ectoderm; notice the *Xsox-2* induction (arrows). (b, l–s) Ventral ectoderm taken from embryos injected with RLDx (magenta in b) and transplanted into stage 13 embryos. The embryos were cultured until stage 17 and analyzed for *Xslug* expression by whole-mount *in situ* hybridization. (l–o) *Xslug in situ* hybridization; (p–s) fluorescent image of the corresponding *in situ* hybridization shown. Graft of a stage 10 (l, p) or 11 (m, q) ectoderm; note the normal *Xslug* expression in both neural folds (l, m) and the *Xslug* expression in the region of the fluorescent transplant (p, q, arrow in l and m). Graft of a stage 12 (n, r) or 13 (o, s) ectoderm; notice the gap of *Xslug* expression in one of the folds (n, o) and the absence of *Xslug* expression in the region of the fluorescent transplant (n, r, o, s). (t) Control of ventral ectoderm taken from a stage 10 embryo, cultured *in vitro* until stage 17, fixed, and analyzed for *Xslug* expression. No expression was detected. (c, u–w) Ventral ectoderm taken from an embryo previously injected with FLDx (green in c) was transplanted into the prospective folds of a stage 11.5 embryo. The embryo was cultured until stage 17, fixed, analyzed for *Xslug* expression, and sectioned. Only the transplant of the stage 13 embryo is shown (u); notice the interruption of the *Xslug* expression in one of the folds (u; arrowhead shows the normal *Xslug* expression) and the absence of *Xslug* expression in the region of the fluorescent transplant (v; in w: asterisk, transplanted ectoderm; arrowheads, host's *Xslug* expression).

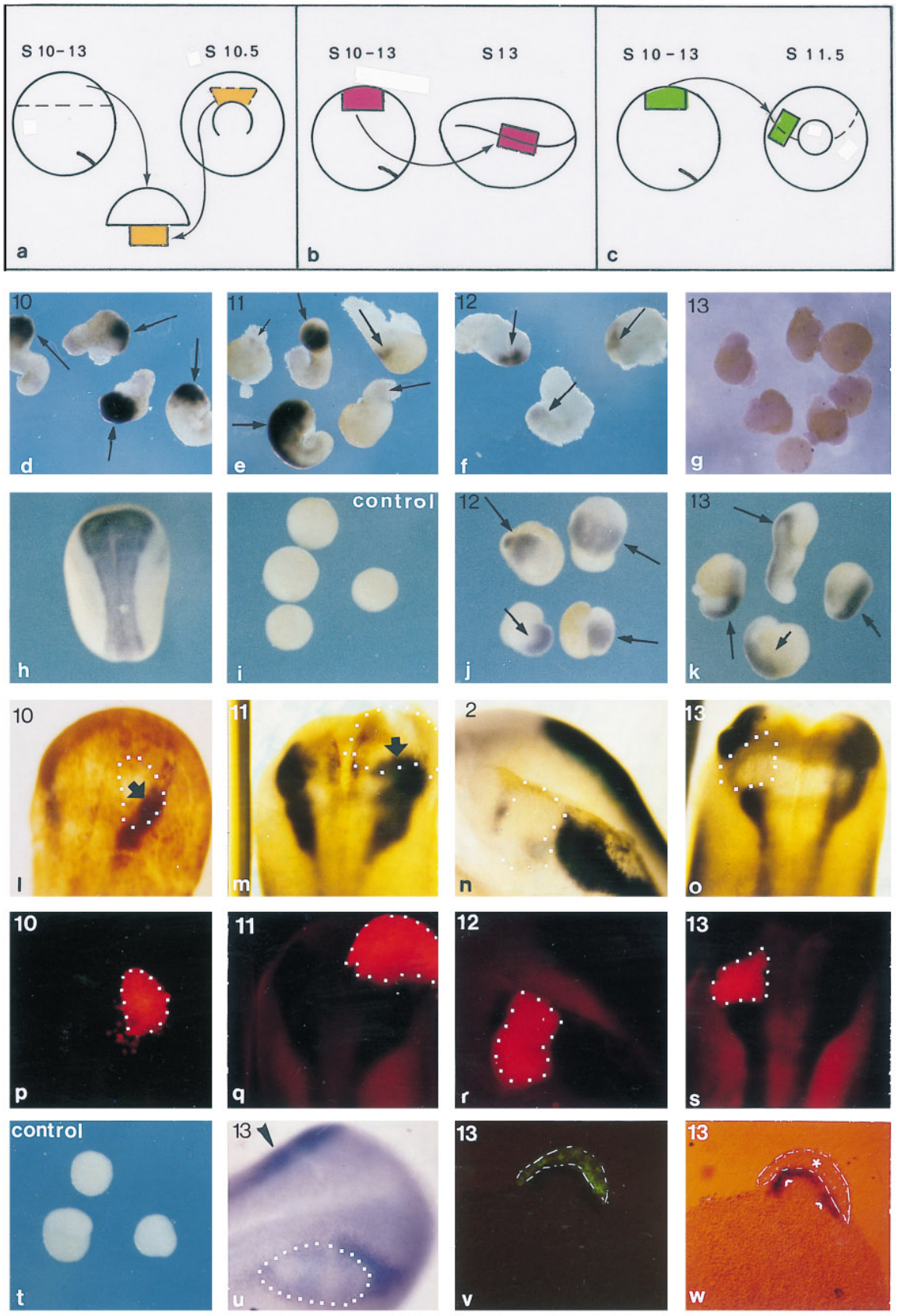
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TABLE 1
Changes in the Competence of the Ectodermal Cells to Respond to Neural Fold Induction

		Cases expressing *Xslug* or *Xsox*/total cases				
		Stage of ectoderm				
Experiment		10	11	12-Dorsal	12-Ventral	13
Conjugate	*Xslug*	31/31	5/5	6/19	7/24	0/10
Conjugate	*Xsox*	11/12	6/6	—	10/13	9/9
Graft St. 13	*Xslug*	3/5	2/8	—	0/7	0/7
Graft St. 11	*Xslug*	6/9	6/16	—	0/13	0/12

from early gastrula embryos expressed *Xslug* after grafting to neural fold regions of a host embryo (Figs. 1l and 1m). Since *Xlsug* expression was found in LRDx-labeled tissue, we conclude that the ectoderm graft is able to respond to neural-fold inducing signals from the host embryo (Figs. 1l, 1m, 1p, and 1q). After older ectoderm was grafted (stage 12, Fig. 1n and stage 13, Fig. 1o), a gap in the stripe of *Xslug* expression was observed, the location of which coincided with that of the fluorescent-labeled graft cells (Figs. 1n, 1o, 1r, and 1s). Together with our *in vitro* analysis, these experiments show that the competence of ectoderm to express *Xslug* in response to signals from the mesoderm is lost after stage 12. We found that the efficiency of induction by the graft method is lower than that by the conjugate method (Table 1, Graft St. 13); we think that this could be due to the poorer healing and slower adhesion observed in the whole embryo compared with the isolated tissues. Since in this set of experiments the hosts were stage 13 embryos, in which the induction had probably already started, it was possible that some additional signals present in earlier embryos were not tested.

In order to analyze earlier signals, in a second set of experiments we used stage 11.5 embryos as hosts (Fig. 1c). Since the neural folds are not visible at this stage, fate maps were used to determine the location of the prospective neural folds/neural crest cells (Mayor *et al.*, 1995). The grafted ectoderm was labeled with LFDx and the host embryo that received the transplant was cultured and analyzed under the same conditions as above, except that the embryos were sectioned to analyze the *Xslug in situ* hybridization and the fluorescence more carefully. The results of these experiments (Table 1, Graft St. 11) were indistinguishable from those obtained with stage 13 hosts (Figs. 1u, 1v, and 1w).

Taken together, the three series of experiments show that the competence of ectoderm to respond to the mesoderm, assessed by the expression of *Xslug*, is lost after stage 12. Furthermore, stage 11.5 and stage 13 hosts show no differences in their ability to induce *Xslug* expression in grafted ectoderm.

The Neural Folds Are Specified after Gastrulation

Since our results suggest that the competence of ectoderm to form neural crest cells in response to mesoderm is lost around stage 12–13, specification of the neural crest must occur prior to this stage. To determine the time at which ectoderm cells are committed to express *Xslug*, we have dissected prospective neural fold ectoderm from stage 10 to 13 embryos, cultured it until an equivalent of stage 17 (Fig. 2a), and scored *Xslug* expression using a sensitive method of *in situ* hybridization (see Materials and Methods). Prospective neural fold explants taken from stage 10 and 11 embryos were unable to express *Xslug* autonomously when cultured until stage 17 (Figs. 2b and 2c), whereas explants taken from stage 13 embryos did express the neural crest marker (Fig. 2d, Table 2). This result suggests that the neural folds are specified to express *Xslug* autonomously at the end of gastrulation, after stage 11 and before stage 13 (Figs. 2b and 2c). Since the level of *Xslug* expression obtained from explants isolated at stage 13 and cultured to stage 17 (Fig. 2d) was never as strong as the normal expression of *Xslug* at stage 17 (Mayor *et al.*, 1995), it is possible that some additional signals are required to augment the initial transcript levels.

An Interaction between Neural Plate and Epidermis Can Induce Neural Folds in the Embryo

There is accumulating evidence supporting a role for neural–epidermal interactions in the genesis of neural crest cells, both in amphibians (Moury and Jacobson, 1989, 1990) and in avian embryos (Dickinson *et al.*, 1995; Liem *et al.*, 1995; Selleck and Bronner-Fraser, 1995). We have therefore conducted experiments to investigate whether neural plate–epidermal interactions can induce the expression of *Xlug*. By using *Xenopus* embryos we had the advantage of being able to inject lineage tracers and make very precise dissections of different tissues in order to test this hypothesis. We grafted anterior neural plate from stage 13 embryos into the lateral epidermis of stage 13 host embryos (Fig. 3a). The host embryo was allowed to heal for about 1 hr in 75% NAM and then transferred to 38% NAM, where it was cultured until a more advanced stage, fixed, and analyzed for *Xslug* expression by whole-mount *in situ* hybridization. A strong ring of *Xslug*-expressing cells was observed at the border of the graft (Fig. 3b). To test which of the two neighboring tissues expresses the neural crest marker, we labeled

the donor embryo at the 1-cell stage with LRDx, thereby labeling the grafted tissue. Our results show that the *Xslug*-expressing cells at the border of the graft are derived from both the (fluorescent-labeled) neural plate (Fig. 3c, arrows in Fig. 3d) and the (unlabeled) epidermis (Fig. 3c, arrowheads in Fig. 3d). Such a strong induction is observed upon grafting anterior neural plate, but not after grafting posterior neural plate (Table 3, Graft), which probably reflects some rostrocaudal differences along the neural plate.

In order to show that the *Xslug* expression is a consequence of neural–epidermal interactions, and not due to some regenerative process similar to that demonstrated in chick embryos (Scherson *et al.*, 1993), we cultured neural plate alone *in vitro* (Fig. 3e). Even after culture to the equivalent of stage 19, the neural plate explant never expressed *Xslug* (Fig. 3f, Table 3, Conjugate).

To eliminate the possibility that *de novo Xslug* expression is a consequence of interactions with the underlying lateral plate mesoderm, we decided to test the inductive ability of this tissue. A piece of anterior neural plate taken from a stage 13 embryo was conjugated with a piece of lateral plate taken from an embryo at the same age (Fig. 3e). The conjugates were cultured until the equivalent of stage 17 and *Xslug* expression was analyzed. No expression was detected in the conjugate (Fig. 3g; Table 3, Conjugate).

Since it has been proposed that neural plate can induce competent ectoderm to differentiate into neural tissue by a homeogenetic signal (Albers, 1987; Servetnick and Grainger, 1991), we decided to test whether this inductive signal could induce neural crest. To this end, we grafted a piece of competent ectoderm from a stage 10, 11, or 12 embryo into the lateral epidermis of a stage 13 embryo (Fig. 3i) and cultured the host embryo as described above. No *Xslug* expression was detected in any of the grafts (Fig. 3j; Table 3, Graft). However, we did detect expression of the neural marker *Xsox-2* in embryos that had received a stage 10 ectoderm transplant (Fig. 3k). These results show that after neural induction, the neural plate can induce additional neural tissue from competent ectoderm but cannot induce neural crest cells.

To prove that an interaction between neural plate and epidermis is sufficient to induce neural crest cells, we cultured tissue recombinants *in vitro*. (Fig. 3e). At a time equivalent to stage 17, *Xslug* transcripts were found in the conjugate (Fig. 3h; Table 3, Conjugate), indicating that no other tissue is required for the expression of *Xslug* at stage 13.

TABLE 2
Time of Commitment to *Xslug* Expression

Stage of ectoderm	10	11	13
Cases expressing *Xslug*/total cases	0/12	2/8	10/13

DISCUSSION

Competence of Ectoderm to Make Neural and Neural Crest Cells

We have previously shown that dorsal mesoderm is able to induce neural crest cells from animal cap ectoderm (Mayor *et al.*, 1995). In the present study, we have used this culture paradigm, along with an *in vivo* approach, to determine the time at which ectoderm is competent to form neural crest cells. We have used *Xslug* expression as a marker for prospective neural crest cells, since transcripts of this gene are restricted to neural crest cells and their neural fold precursors at early stages of development. Our results indicate that the ventral ectoderm is able to respond to *Xslug*-inducing signals from dorsal mesoderm until approximately stage 12. In contrast, stage 13 neural plate (but not neural plate from younger embryos) grafted into a field of prospective epidermal cells of a stage 13 host is able to induce the expression of *Xslug*, indicating that such ventral ectoderm is able to respond to inducing signals from neural plate until later stages of development.

Our experiments indicate that the competence of ectoderm to express *Xsox-2*, a neural marker, lasts until stages 14–15 while ectoderm's ability to express Xslug persists only until stage 12. These results suggest that the induction of the neural plate is not enough to induce neural crest. The changes in the competence to express *Xsox-2* described by us are similar to those described for the XLHbox 6 gene (Sharpe *et al.*, 1987) and N-CAM (Servetnick and Grainger, 1991).

Commitment of Ectoderm Cells to a Neural Crest Fate

When do (prospective) neural fold cells become committed to their fate? To address this question, we have cultured neural fold explants and assayed for the presence of neural crest cells in the isolates by *in situ* hybridization with a probe to *Xslu*. Our results confirm our previous study using the less-specific neural crest marker *Xsna* (Mayor *et al.*, 1995). We find that the neural folds are specified at the end of gastrulation. Therefore, the neural folds are specified during the same period of development in which the ectoderm loses the competence to respond to neural fold induction. In addition, our results show that the intensity of the signal reached in the ectoderm cultured *in vitro* is weaker than the *Xslug* expression observed in the whole embryo. These observations suggest that the induction of the neural fold is not completed at the end of gastrulation and that this tissue is only partially specified.

Induction of Neural Crest Cells by Neural Plate–Epidermal Interactions

There is evidence from experiments in axolotl that the neural folds, and some neural crest derivatives such as mela-

TABLE 3
Induction of *Xslug* by Neural Plate/Epidermis Interaction

Experiment	Cases expressing *Xslug*/total cases
Graft	
Anterior neural plate into epidermis	18/35
Posterior neural plate into epidermis	2/28
Stage 10 animal cap into epidermis	0/10
Conjugate	
Neural plate alone	0/14
Neural plate/lateral mesoderm	0/15
Neural plate/epidermis	5/12

nocytes, are induced by an interaction between neural plate and epidermis (Moury and Jacobson, 1989, 1990). This conclusion is based on experiments in which a graft of albino neural plate was transplanted into ventral epidermis of a wild-type embryo; the authors identified the induction of neural folds by morphological criteria and the neural crest by the appearance of melanocytes. Equivalent experiments have been carried out in chicken, showing that the neural plate can interact with nonneural ectoderm to induce neural crest (Dickinson *et al.*, 1995; Sechrist *et al.*, 1995; Selleck and Bonner-Fraser, 1995). In the present study, we have extended these studies to *Xenopus* and show that an interaction between neural plate and epidermis is sufficient to induce neural crest cells, both in the embryo and *in vitro*. We have also demonstrated that the induced neural crest cells arise from both the neural and epidermal tissue, supporting the findings of previous studies (Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995). We have extended our analyses by eliminating the following alternatives as possible sources of inductive signal: a homeogenetic signal coming from the host neural plate, the ventral mesoderm underlying the graft, and the regeneration of the neural crest from the dissected neural plate.

Models of Neural Crest Cells Formation

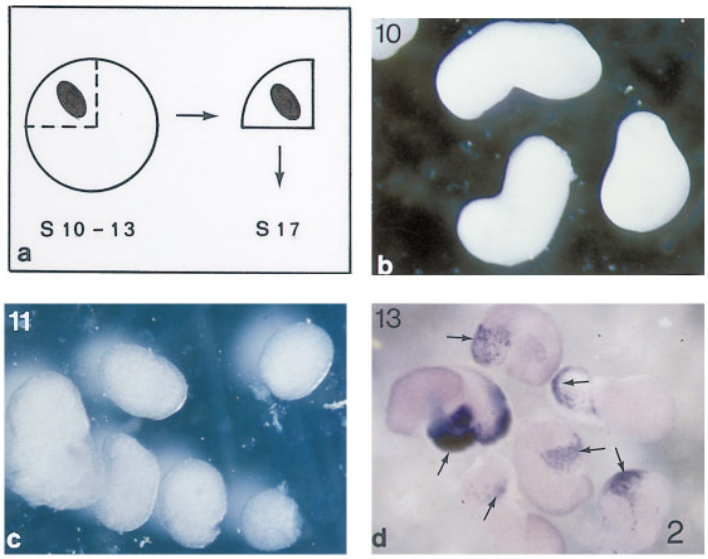
If neural crest cells are generated by neural–epidermal interactions exclusively, one would expect to find neural crest cells only in the presence of neural tissue. However, Raven and Kloos (1945) have shown that it is possible to induce neural crest derivatives in the absence of neural plate, and Holtfreter and Hamburger (1955) claim that neural crest cells develop more readily than neural plate in their induction experiments. Our own experiments indicate that the lateral mesoderm from an early gastrula is a strong neural crest inducer but is a poor inducer of neural plate (Mayor *et al.*, 1995). These three examples lend support to the idea that formation of neural plate and the generation of neural crest cells are independent events (Nieuwkoop *et al.*, 1985).

Two recent molecular manipulations also bear on this issue. Overexpression of the *Xenopus* proneural gene homologue *Xash* and expression of a dominant-negative mutation of the neurogenic gene *Notch* both produce an increase in the size of the neural plate (Coffman *et al.*, 1993; Turner and Weintraub, 1994; Zimmerman *et al.*, 1993). If neural crest cells are generated by neural plate–epidermal interactions exclusively, we would expect the same number of neural crest cells to be generated in both cases, but at a more lateral position in the embryo. In contrast, the neural plate seems to enlarge at the expense of neural crest and epidermis.

One model that can explain these findings is that of Albers (1987), who proposed that changes in the competence of ectoderm to respond to a combined neural/neural crest inducer are responsible for determining the location of neural crest cell precursors (see the Introduction). In support of this model, Servetnick and Grainger (1991) have shown that homeogenetic signals can spread from the neural plate. Our

FIG. 2. Specification of *Xslug* in the neural crest. (a) Animal cap explants containing the prospective neural crest, dissected at different stages (b, 10; c, 11; and d, 13) were cultured until the equivalent of stage 17, fixed, and analyzed for *Xslug* expression. Notice the absence of *Xslug* expression when the ectoderm is dissected before stage 12 (b, c) and the visible *Xslug* expression when the ectoderm is taken from a stage 13 embryo (arrows in d).

FIG. 3. Induction of the neural crest by an interaction between neural plate and epidermis. (a–d) A piece of anterior neural plate taken from a stage 13 embryo previously injected with RLDx was transplanted to the ventral region of a normal stage 13 embryo. The host embryo was cultured until stage 23 (b) or 17 (c, d) and fixed and the *Xslug* expression was analyzed. (b) Upper embryo: control showing the normal *Xslug* expression in the neural fold. Lower embryo shows the expression in a ring around the graft (arrow). Fluorescence (c) and *Xslug in situ* hybridization (d) of the same embryo; notice that the induced crest cells that express *Xslug* at the border of the graft come from the fluorescent neural plate (arrows in d) and from the unlabeled epidermis (arrowheads in d). Some RLDx-labeled cells have left the graft but they do not express *Xslug*. (e–h) Pieces of different tissues were dissected from a stage 13 embryo and conjugated with another tissue or cultured *in vitro* until the equivalent of stage 17. Then, they were fixed and *Xslug* expression was analyzed. (f) Neural plate cultured alone never expressed *Xslug*. (g) A conjugate of neural plate and lateral mesoderm never expressed *Xslug*. (h) A conjugate of anterior neural plate and lateral epidermis showed a strong *Xslug* expression (arrows). (i–k) A piece of stage 10 animal cap was grafted into the lateral region of a stage 13 embryo. The embryo was cultured until stage 17 and *Xslug* (j) and *Xsox-2* (k) expression was analyzed by whole-mount *in situ* hybridization. The dotted line surrounds the transplant; notice that *Xsox-2* (k), but no *Xslug* (l) expression was detected in the graft. np, anterior neural plate; ep, epidermis; m, lateral plate mesoderm.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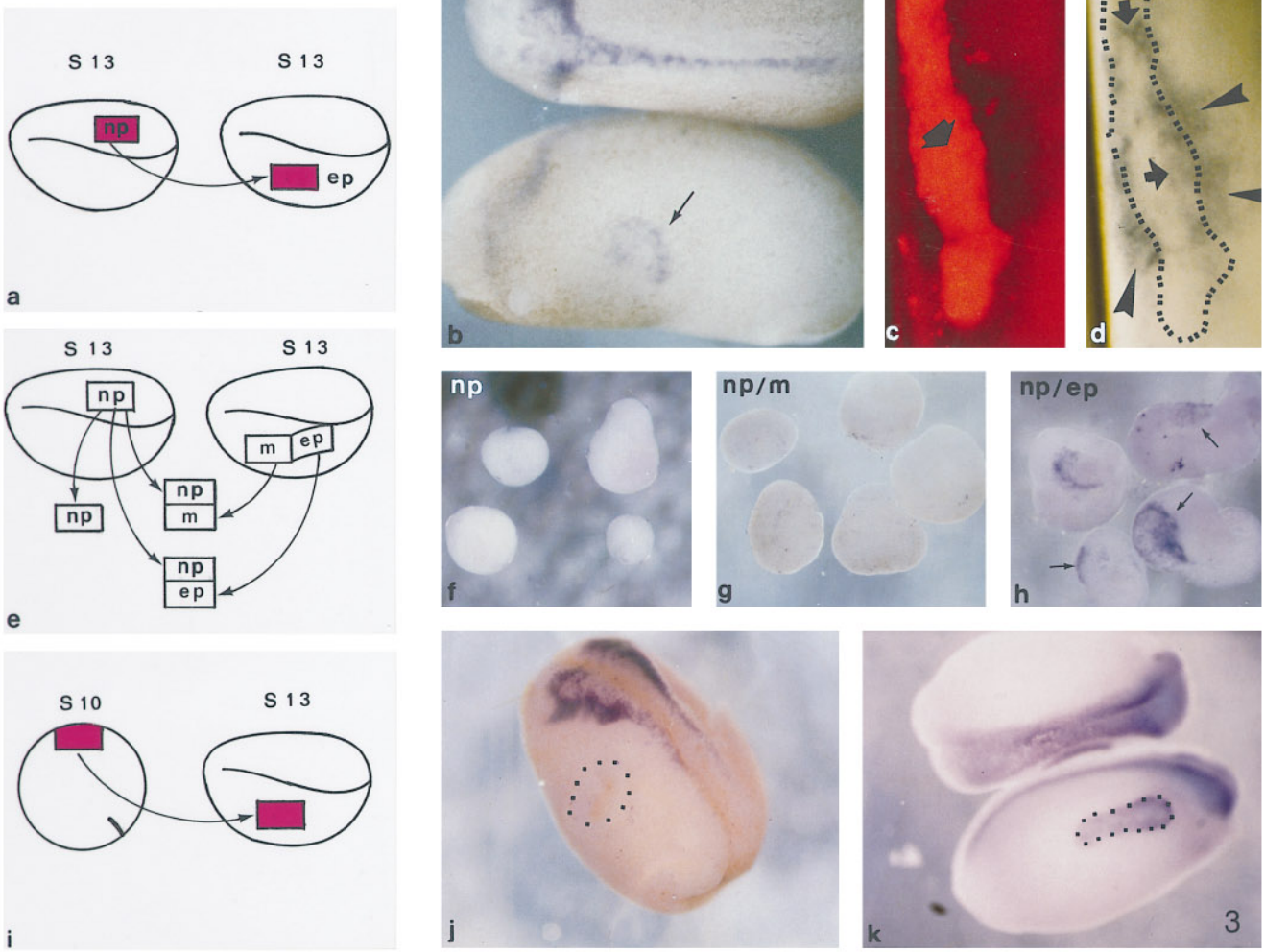
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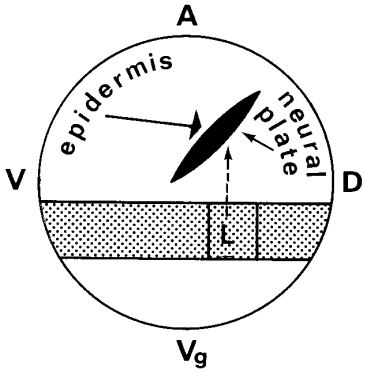

FIG. 4. Model of neural crest induction in *Xenopus.* We propose that during gastrulation a hypothetical signal coming from the lateral mesoderm (dashed line from L) starts the induction of the neural crest. At the same time the neural plate is induced. Later, at the end of gastrulation, the induced neural plate interacts with the epidermis to complete the induction of the neural crest. Dotted area: mesoderm. Black area: prospective neural crest. A, animal pole; Vg, vegetal pole; D, dorsal side; V, ventral side.

own observations also support this model. Young (stage 10) ectoderm will express the neural marker *Xsox-2* when grafted into a field of (stage 13) epidermis, showing that (a) neural-inducing signals are present in this region of the embryo, and (b) stage 10 ectoderm is able to respond to the signal. As expected, the (older) host ectoderm neighboring the graft fails to express *Xsox-2.* If Albers' model is correct, we would expect older ectoderm (from stage 11–12 embryos) with an ''intermediate competence'' to express *Xslu* after grafting to a similar position. In contrast, we failed to find evidence of neural crest cells after grafting stage 11 or 12 ectoderm. Additionally, we do not find that *Xsox-2*-expressing grafted ectoderm is able to interact with the surrounding epidermis to induce neural crest cell formation. These apparent contradictory results can be explained in two ways. It is possible that the induction of the neural plate by a homeogenetic signal takes a long time, and during this period the ectoderm loses the competence to form neural crest. Alternatively, it is possible that the neural plate induced by the homeogenetic signal has some characteristics that make it unable to interact with the epidermis to induce neural crest. We have shown that only the anterior neural plate can induce neural crest by an interaction with the epidermis. The posterior neural plate does not have this ability and the homeogenetic signal could induce some kind of posterior neural plate.

We propose the following model to explain the induction of the crest in *Xenopus* (Fig. 4). In the first step, a signal coming from the lateral mesoderm (dashed line) and possibly from the dorsal mesoderm induces the neural plate and, by an unknown mechanism, the neural fold at the border of the neural plate. This step occurs during gastrulation and induces a weak level of expression of *Xslug* and *Xsna.* Once gastrulation has ended, a second step takes place: the induced neural plate interacts with the surrounding epidermis through the weakly induced neural folds to reinforce the first induction (thick lines). This produces an increase in the level of *Xsna* and *Xslug* and possibly a sharpening of the limits of the neural folds. The competence of ectoderm to respond to the mesoderm is lost at stage 12, before the neural plate can interact with the epidermis, suggesting that two different signals are involved in the induction of the neural folds. However, our results do not allow us to discard absolutely the alternative hypothesis that the neural crest is induced just by an interaction between neural plate and epidermis.

What is the molecular basis of neural crest formation? It has been recently proposed that neural folds are induced by the combined effects of dorsal signals, such as Noggin, and ventral signals, such as FGF, *Xwnt-8,* or BMP-4 (Mayor *et al.,* 1995; Liem *et al.,* 1995). The ability of Noggin to induce neural tissue has been clearly shown (Lamb *et al.,* 1993) and there is some evidence to suggest that FGF may also play a role in neural induction (Kengaku and Okamoto, 1993, 1995; Lamb and Harland, 1995; Cox and Hemmati-Brivanlou, 1995). BMP-4 has been shown to induce ventral and posterior mesoderm (Dale *et al.,* 1992; Graff *et al.,* 1994; Maeno *et al.,* 1994; Schmidt *et al.,* 1995) and there is some evidence to suggest that Noggin and BMP-4 have opposing actions: embryos that express a dominant negative form of the BMP-4 receptor (and so knock out BMP-4 activity) show a phenotype very similar to Noggin overexpression (Schmidt *et al.,* 1995; Mayor *et al.,* 1995). We are currently performing experiments aimed at defining a more precise role of these molecules in the induction of the neural crest cells.

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